Morphogenic Role for Acetylcholinesterase in Axonal Outgrowth during Neural Development

John W. Bigbee,[1] Karun V. Sharma,[1] Jyotsna J. Gupta,[2] and Jeffrey L. Dupree[3]

[1]Department of Anatomy, Virginia Commonwealth University Medical Campus, Richmond, Virginia; [2]Department of Physical Therapy, [3]Brain Developmental Research Center, University of North Carolina, Chapel Hill, North Carolina

Acetylcholinesterase (AChE) is the enzyme that hydrolyzes the neurotransmitter acetylcholine at cholinergic synapses and neuromuscular junctions. However, results from our laboratory and others indicate that AChE has an extrasynaptic, noncholinergic role during neural development. This article is a review of our findings demonstrating the morphogenic role of AChE, using a neuronal cell culture model. We also discuss how these data suggest that AChE has a cell adhesive function during neural development. These results could have additional significance as AChE is the target enzyme of agricultural organophosphate and carbamate pesticides as well as the commonly used household organophosphate chlorpyrifos (Dursban). Prenatal exposure to these agents could have adverse effects on neural development by interfering with the morphogenic function of AChE. — *Environ Health Perspect* 107(Suppl 1):81–87 (1999). *http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/81-87bigbee/abstract.html*

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Accumulating evidence indicates that acetylcholinesterase (AChE) has extrasynaptic functions during neural development (*1–3*). This idea was initially based on *in vivo* observations that AChE is transiently expressed by neurons throughout periods of axonal outgrowth prior to synaptogenesis, a period during which the classical cholinolytic role for AChE in terminating nervous transmission is unnecessary. For example, in the central nervous system (CNS), Robertson and colleagues (*3,4*) have demonstrated transient AChE activity in thalamic neurons at a time when their axons are growing into the cerebral cortex. Similar results have been reported by Kristt (*5*) in rat and by Kostovic and colleagues in developing primates (*6*). This expression of AChE has also been confirmed at the messenger RNA level by *in situ* hybridization (*7*). In the chick, transient AChE expression occurs in developing spinal cord neurons, which coincides with axonal outgrowth from these cells (*1,8,9*). In the peripheral nervous system (PNS), AChE is transiently expressed by developing dorsal root ganglion (DRG) neurons (*10–14*) and later in their axons and growth cones in the spinal cord (*15,16*). Together, these data strongly suggest that AChE plays a developmental role in the morphogenesis of the nervous system.

Our laboratory has examined this developmental expression of AChE in primary cultures of DRG neurons that are noncholinergic, yet express high levels of AChE during neurite outgrowth. This article summarizes our results and discusses possible mechanisms by which AChE may effect its growth-promoting action.

Materials and Methods

Preparation of Dorsal Root Ganglion Neuronal Cultures

DRG neuronal cultures were prepared from E-15 rat embryos as previously described (*17–22*). Neurons were plated either onto a substratum of type I collagen or Matrigel (Becton Dickinson and Co., Franklin Lakes, NJ) and maintained in Eagle's minimal essential medium supplemented with 10% NuSerum, 0.3% additional glucose, and crude nerve growth factor at 37°C with 5% CO_2. Matrigel is an artificial basal lamina extract that contains laminin, type IV collagen, and entactin and forms a highly permissive substratum for neurite growth. Cells were plated in the center of 35-mm culture dishes and extended radially oriented neuritic processes from this central plating area. The care and treatment of experimental animals were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee, Division of Animal Resources, Virginia Commonwealth University (*23*).

Pharmacologic Inhibitor Treatment

After plating, cultures received either 1,5-bis-(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW284c51) or physostigmine at a concentration ranging from 10^{-4} M to 10^{-7} M or fresh medium only (*18*). BW284c51 is a bis-quaternary nitrogen compound that is a highly specific inhibitor for AChE. Because of this specificity, BW284c51 is routinely used to confirm AChE activity in both histochemical and biochemical determinations. Physostigmine is a naturally occurring carbamate inhibitor of AChE. These two compounds were used because they inhibit AChE activity by different mechanisms. Medium with or without inhibitors was changed daily. BW284c51 is used to confirm AChE activity and as a control for inhibitor treatment, the butyrylcholinesterase (BChE) inhibitor tetraisopropyl pyrophosphoramide (iso-OMPA) substituted for either BW284c51 or physostigmine. BChE was not expressed in DRG neurons either at the developmental stage when they were removed from the embryo (*14*) or after the cells were placed in culture (*18*). Cultures were maintained for 14 days and then examined by darkfield microscopy to determine the extent and pattern of outgrowth and by transmission electron microscopy to reveal ultrastructural changes. In addition, to assess the distribution of neurofilaments, the major cytoskeletal component of neurons, cultures were examined by immunofluorescence microscopy as previously described (*19,20*), using a monoclonal antibody to the low molecular weight neurofilament subunit protein NF68.

Monoclonal Antibody Treatment

Cultures were treated with an AChE monoclonal antibody, MAB304 (Chemicon Int'l. Inc., Temecula, CA), using either an

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Address correspondence to J. W. Bigbee, Dept. of Anatomy, VCU Medical Campus, PO Box 980709. Richmond, VA 23298-0709. Telephone: (804) 828-0948. Fax: (804) 828-9477. E-mail: jbigbee@gems.vcu7.edu

Abbreviations used: AChE, acetylcholinesterase; BChE; butyrylcholinesterase; BW284c51, 1,5-bis-(4-allyldimethylammoniumphenyl pentan-3-one dibromide; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; DFP, di-isopropylfluorophosphate; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; isoOMPA, tetraisopropyl pyrophosphoramide; PNS, peripheral nervous system.

acute high-dose or a chronic low-dose exposure protocol as previously described (*22*). For the acute treatment, 10-day-old cultures were exposed to 200 µg/ml of either MAB304 or normal mouse IgG, or medium without antibody for 3 hr. For the chronic studies, 4-day-old cultures received medium containing 20 µg/ml of either MAB304 or normal mouse IgG or medium without antibody for 6 days. The area of neurite outgrowth was calculated using a computer-generated perimeter that extended around the distalmost extent of the neurites. A representative set of chronically treated cultures was also examined by scanning electron microscopy.

AChE Assays

AChE activity was detected histochemically as previously described (*18,19,21*), using acetylthiocholine as the substrate analog according to the modification of the method of El Badawi and Schenk (*24*). AChE activity was quantified either by colorimetric analysis based on the method of Ellman et al. (*25*), using acetylthiocholine, or by radiometric assay, using tritiated acetylcholine, according to the method of Hall (*26*) as we have previously described (*18,21*).

Results

The Level of AChE Expression Correlates with Neurite Outgrowth

Cultured DRG neurons showed a developmental increase in AChE that paralleled the extent of neurite outgrowth (Figure 1). AChE expression increased 5-fold between 3 and 7 days in culture (*17,18*), which is consistent with the developmental expression of AChE by DRG neurons *in vivo* (*10,14*).

Pharmacologic Inhibitor Treatment Reduces Neurite Outgrowth

DRG neurons displayed a dose-dependent reduction in outgrowth in the presence of either BW284c51 or physostigmine (Figure 2). No effect on outgrowth was observed when iso-OMPA was substituted for either of these inhibitors. With increasing doses the outgrowth decreased and appeared more fasciculated than in control cultures. For both compounds, at the highest doses tested, minimal neurite outgrowth was observed beyond the central plating area. These results were not due to differential cell survival as no difference in cell number was found between control and treated cultures at the completion of the experiments. Furthermore, this effect was reversible as additional outgrowth occurred and appeared normal after removal of either inhibitor. The inhibitor concentrations used in these studies were consistent with values reported in the literature (*27*) for inhibition of AChE *in vitro*. The results clearly indicate that the level of AChE inhibition closely correlated with the extent of decreased outgrowth (Figure 3). Interestingly, co-administration of 1 mM dibutyryl cyclic adenosine monophosphate (cAMP) along with BW284c51 significantly increased both neurite outgrowth and AChE expression compared with inhibitor treatment alone (*20*).

The esteratic activity of AChE is used as a convenient and accurate reporter for both the cellular localization and level of AChE expression. However, it is important to note that the observed effects of AChE inhibitors on neurite outgrowth are independent of the ability of these compounds to inhibit esteratic activity. For example, we and others (*20,28,29*) have shown that irreversible inhibition of AChE with the organophosphate diisopropylflurophosphate (DFP) has no effect on neurite outgrowth from either CNS or PNS neurons. These studies indicate that the catalytic activity of AChE is not required for its

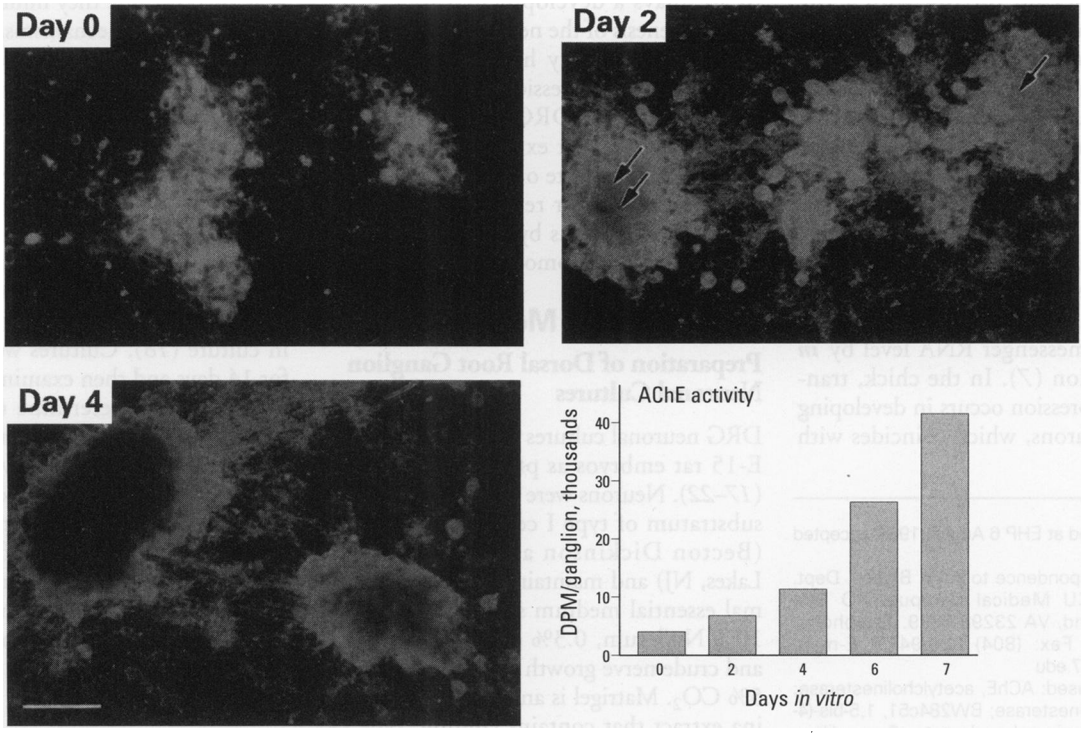

Figure 1. AChE activity increases with neurite outgrowth. Darkfield photomicrographs of DRG cultures histochemically stained for AChE activity at day 0, day 2, and day 4 after plating. Small clusters of cells within the plating area express detectable levels of AChE activity at day 2 (arrows) which becomes prominent by day 4 as a dark reaction product. This activity increases in both the cell bodies and neurites with time in culture. Quantitation of the AChE activity is shown in the lower right panel using a radiometric assay (*21,25*). Bar = 150 µm for all panels.

morphogenic function in neurite growth. Furthermore, not all AChE inhibitors have the equivalent effect on neurite outgrowth (Table 1), suggesting that some other physicochemical property of these molecules accounts for their "neuritostatic" ability. On the basis of these data, Layer and colleagues (*30*) have proposed the secondary site hypothesis. This premise states that some, as yet undefined, secondary site on the AChE molecule is responsible for its growth-related properties. The ability to interfere with this site could therefore depend on the biochemical properties of the inhibitor molecule, including its shape and charge.

Pharmacologic Inhibitor Treatment Leads to Alterations in the Neuronal Cytoskeleton

Along with the retarded neurite outgrowth, 40 to 50% of the neuronal cell bodies treated with BW284c51 show large accumulations of neurofilaments (*18–20*) (Figure 4A). At the ultrastructural level, these masses are composed of 10-nm filaments (Figure 4B) and resemble cytoplasmic inclusions present in neurons in cases of cortical atrophy in the CNS and ganglioneuroma in the PNS (*31*). In our model the accumulation of neurofilaments could be due to a direct effect on their processing and transport, leading to impaired outgrowth. Alternatively, AChE inhibitor treatment could directly perturb neurite extension and elongation, leading to a secondary backup of cytoskeletal elements in the cell body.

AChE Monoclonal Antibody Produces Neurite Detachment and Altered Neurite Outgrowth

On the basis of the results from our studies using pharmacologic inhibitor treatment, we examined the effects of a monoclonal anti-AChE antibody, MAB304, on neurite outgrowth and attachment (*22*). Because this antibody does not inhibit enzyme activity (*22,32*), yet causes the morphologic and adhesive changes, its reactive epitope may be related to the secondary, growth-related site on AChE. We first determined that the antibody recognized AChE by immunocytochemical and enzyme-linked immunosorbent assay (ELISA) studies (*22*). We also confirmed that AChE is on the cell surface and that antibody binding occurred in unfixed cultures (*22*). The latter result ensured that the antibody binds AChE under the experimental conditions used for the perturbation studies. As shown in Figure 5, when cultures that had been allowed to extend neurites were exposed to a high concentration of MAB304 antibody, the distal tips of the neurites detached within 90 min. Upon removal of the antibody, new growth was observed within 8 to 12 hr. In experiments similar to the pharmacologic

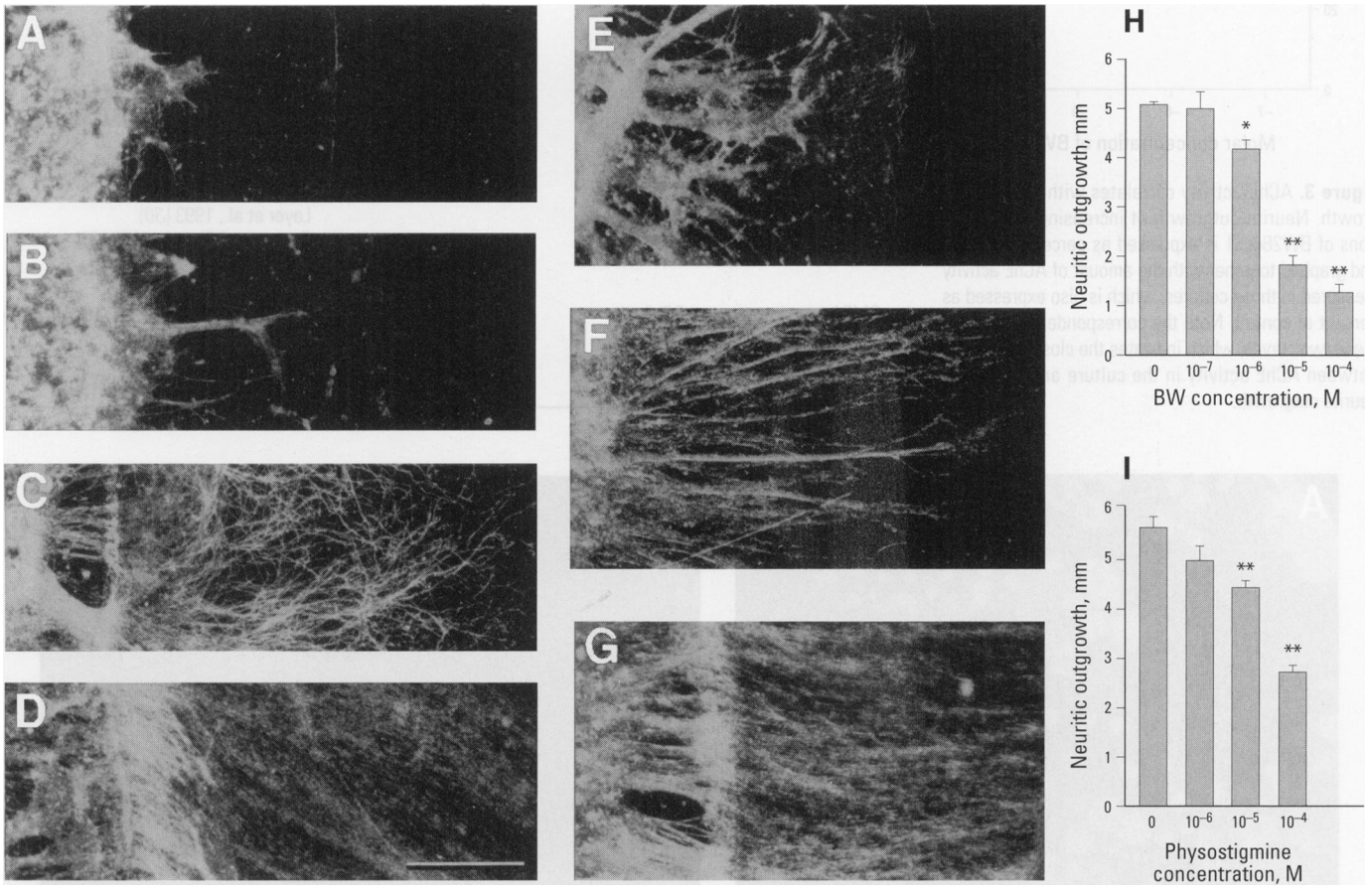

Figure 2. BW284c51 and physostigmine retard neurite outgrowth. Abbreviation: BW, BW 284c51. Panels *A* through *G* are darkfield micrographs of unstained DRG neuron cultures grown for 10 days in medium containing either BW284c51 (*A*, 10^{-4} M; *B*, 10^{-5} M; *C*, 10^{-6} M) or physostigmine (*E*, 10^{-4} M; *F*, 10^{-5} M) or medium only (*D* and *G*). In each panel, the central plating area containing the cell bodies is to the left and neurites extend to the right. In both cases, there is a dose-dependent retardation of outgrowth and an increase in neurite fasciculation, These results are shown graphically in panels *H* and *I*. Results are expressed as means ± SEM. Statistics for differences among the means were carried out by the Students *t*-test. (*$p<0.01$; **$p<0.001$). Bar = 2 mm for panels *A–G*.

inhibitor studies described above, cultures exposed to low AChE antibody concentrations for 6 days had a 50% reduction in the area of neurite outgrowth, compared to treatment with normal mouse IgG and untreated controls (Figure 6). In addition, the neurites become more densely packed, highly interlaced, and their distal ends terminate as a uniform growth front. Growth cones are mostly confined to this leading edge and are larger than those in either control group. Substitution of immunodepleted MAB304 ascites fluid eliminates both the detachment of neurites and retarded outgrowth, indicating that the effects are due to antibody in the ascites fluid.

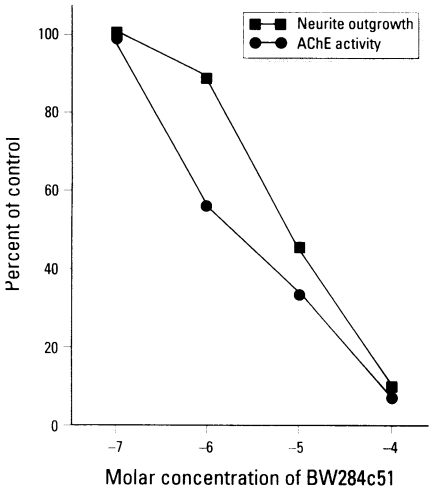

Figure 3. AChE activity correlates with neurite outgrowth. Neurite outgrowth at increasing concentrations of BW284c51 is expressed as percent of control and graphed together with the amount of AChE activity measured in those cultures, which is also expressed as percent of control. Note the correspondence between these two curves, which indicates the close correlation between AChE activity in the culture and degree of neurite outgrowth.

Level of AChE Expression Is Modulated by the Extracellular Matrix

A potential functional interaction between AChE and components of the extracellular matrix is suggested by our results, which show that AChE levels can be altered by DRG neurons when they encounter an extracellular environment that varies in its degree of permissiveness to outgrowth (*21*). As shown in Figure 7, DRG neurons grown on a substratum of type I collagen exhibit neurites that are more fasciculated and less extensive than those on the more permissive Matrigel substratum. Furthermore, the neurons on type I collagen express approximately twice as much AChE as cells grown on Matrigel, suggesting that more AChE is required for growth on a less permissive substratum.

Discussion

Our studies using cultured DRG neurons show that AChE levels increase in parallel with neurite outgrowth and that this expression can be modulated in response to the type of substratum. Furthermore, certain AChE inhibitors produce a dose-dependent, but reversible, reduction in neurite outgrowth (*5,15,17*), which is accompanied by

Table 1. Comparison of target specificity and effect on neurite outgrowth of cholinesterase inhibitors.

Compound	Enzyme target	Effect on outgrowth	Reference
BW284c51	AChE	++++	Bataille et al., 1998 (*38*) Bigbee and DeVries, 1987 (*17*) Dupree and Bigbee, 1994, 1996 (*18,20*) Jones et al., 1995 (*29*) Layer et al., 1993 (*30*) Small et al., 1995 (*28*) Srivatsan and Peretz, 1997 (*39*)
Physostigmine	AChE	++	Dupree and Bigbee, 1996 (*20*)
Edrophonium	AChE	+	Small et al., 1995 (*28*) Srivatsan and Peretz, 1997 (*39*)
Ecothiophate	AChE/BChE	—	Jones et al., 1995 (*29*) Layer et al., 1993 (*30*)
DFP	AChE/BChE	—	Dupree and Bigbee, 1996 (*20*) Jones et al., 1995 (*29*) Small et al., 1995 (*28*)
iso-OMPA	BChE	—	Dupree and Bigbee, 1994, 1996 (*18,20*) Jones et al., 1995 (*29*) Layer et al., 1993 (*30*) Srivatsan and Peretz, 1997 (*39*)

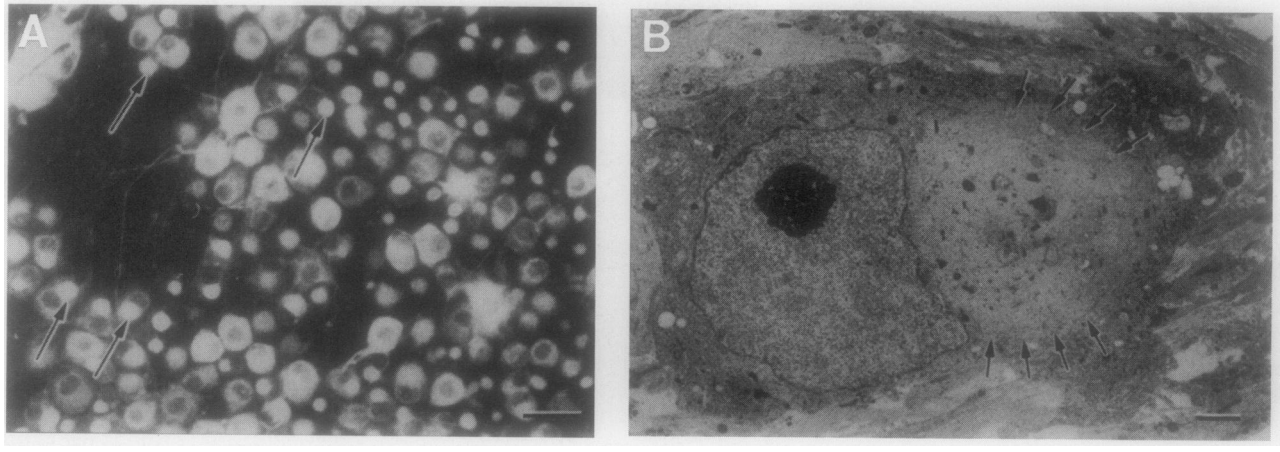

Figure 4. AChE inhibitor treatment produces neurofilament accumulations. DRG neurons were grown for 10 days in the presence of BW284c51 at 10^{-4} M concentration and examined by immunofluorescence (*A*) and transmission electron microscopy (*B*). In *A*, a monoclonal antibody to the low molecular weight protein subunit of neurofilaments reveals a large accumulation of immunoreactive material in nearly half of the cell bodies (arrows). Electron microscopy (*B*) reveals that these masses are composed almost entirely of neurofilaments with a few trapped organelles (arrows). Bar in *A* = 50 µm; bar in *B* = 2.0 µm.

accumulations of neurofilaments in the cell body (*15–17*). Finally, exposure to an AChE monoclonal antibody decreases neurite outgrowth as well as altering the morphology of that outgrowth (*22*). Collectively, these data strongly support a morphogenic role for AChE in neurite outgrowth. Moreover, our findings that AChE antibody treatment can also cause rapid detachment of neurites from the substratum (*22*) further suggest that AChE functions by an adhesive mechanism. We are currently attempting to identify the reactive epitope for MAB304 on the AChE molecule and to determine the proximity of that site to the opening of the active site gorge. In doing so we may be able to determine if the perturbation in neurite outgrowth resulting from anti-AChE antibody treatment involves a site related to, or more distant, from the site(s) occupied by pharmacologic agents, i.e., BW284c51 or physostigmine.

The direct involvement of AChE in process outgrowth has been demonstrated

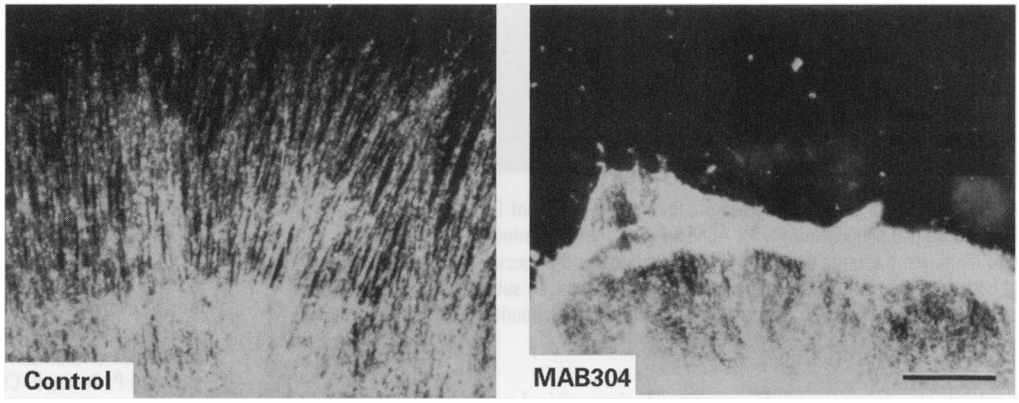

Figure 5. Short-term, high-dose antibody treatment causes neurite detachment. Ten-day-old cultures were treated for 1–3 hr with 200 µg/ml of MAB304. The darkfield micrograph of the untreated culture (left panel) shows the radially oriented pattern of neurite outgrowth with the central plating area beyond the bottom of the field. Within 2 hr of antibody addition (right panel) the distal neurites had detached and continued to detach centripetally with time. Removal of the antibody resulted in resumed neurite outgrowth within 8–12 hr. No complement activity was detected in the culture medium. Bar = 1 mm in both panels.

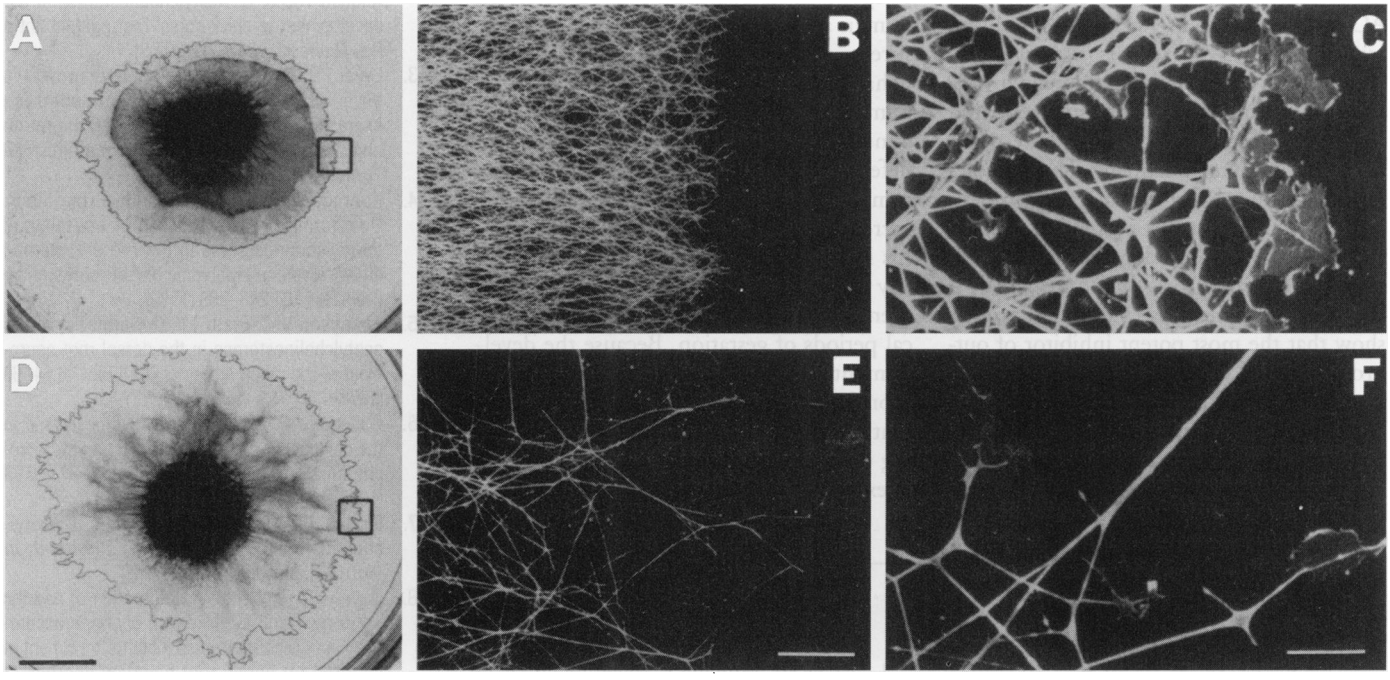

Figure 6. Long-term, low-dose antibody treatment reduces neurite outgrowth. DRG neuron cultures were treated for 6 days with either 20 µg/ml MAB304 (*A–C*) or normal mouse IgG (*D–F*). Panels *A* and *D* are low magnification images of an entire culture showing the central plating area containing the DRG neuronal cell bodies and radially oriented neurite outgrowth. The extent of the outgrowth is indicated by a computer-generated perimeter that was used to calculate the total outgrowth area. Antibody treatment resulted in a 50% reduction in outgrowth based on measurements from a minimum of 12 cultures obtained from three independent experiments. Panels *B* and *E* are low magnification scanning electron micrographs taken from the outgrowth margins indicated by the boxes in *A* and *D*. Note that MAB304 treatment (*B*) produced a more densely interlacing pattern of outgrowth that terminates at a very uniform leading edge compared with normal IgG control (*E*). In addition, the growth cones are larger and restricted to the outgrowth margin in antibody-treated cultures (*C*). Bar in *D* = 0.5 cm for panels *A* and *D*; bar in *E* = 250 µm for panels *B* and *E*; bar in *F* = 25 µm for panels *C* and *F*. Reproduced from Bigbee and Sharma (*46*) with permission from Plenum Press.

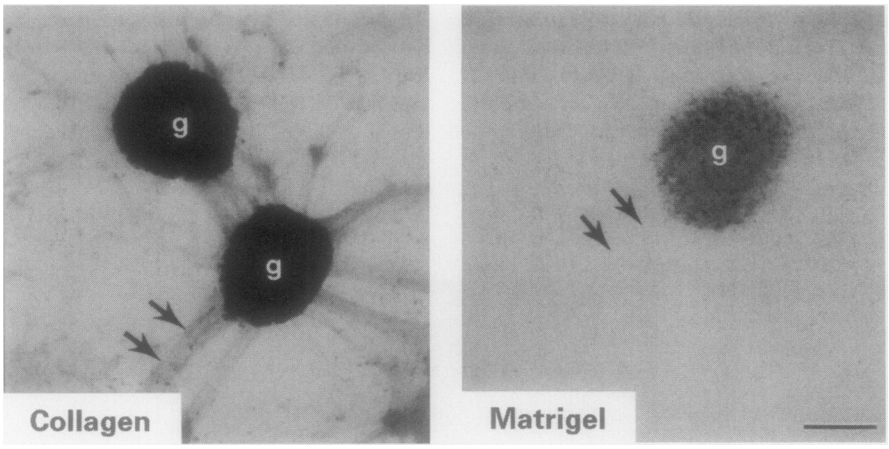

Figure 7. Effects of culture substratum on AChE expression. Photomicrographs of DRG grown for 4 days on collagen type I or Matrigel and stained histochemically for AChE activity. These experiments were performed with nondissociated ganglia to eliminate the large amount of cellular migration that occurs on Matrigel. Note the intense, dark staining of both the ganglia (g) and neurites (arrows) on the collagen substratum, which contrasts with the lower activity seen on Matrigel. AChE activity is barely detectable in the neurites (arrows). Bar = 350 μm in both panels.

in recent studies in which the expression of AChE was genetically manipulated. Overexpression of AChE in cell lines (*33,34*), spinal neurons (*35*), or retinal cells (*36*) caused increased outgrowth that paralleled the level of AChE expression. Conversely, decreasing the expression of AChE, using antisense techniques, reduced outgrowth (*34,37*).

Other *in vitro* studies employing a variety of neuronal cell types have reported retardation of neurite outgrowth in response to AChE inhibitor treatment. These studies used chick tectal and retinal ganglion neurons (*30*), rat sympathetic ganglion neurons (*28*), dopaminergic midbrain neurons (*29*), spinal motor neurons (*38*), and *Aplysia* pedal ganglion neurons (*39*). These studies show that the most potent inhibitor of outgrowth was BW284c51, which occupies both the catalytic and the peripheral anionic sites of the active center gorge of AChE. On the basis of the crystallographic data for AChE complexed with the AChE inhibitor decamethonium, which is also a linear, bisquaternary nitrogen compound (*40*), it is likely that the BW284c51 molecule extends beyond the opening of the gorge. In this orientation, BW284c51 could interfere with the proposed secondary growth-related site (*30*). Thus, examination of the physicochemical properties of AChE inhibitors may provide additional clues about the location and/or nature of this secondary site on AChE.

Agricultural and household pesticides that target AChE could interfere with this noncholinergic role of AChE if exposure occurs during critical periods of nervous system development. As indicated earlier, DFP has no effect on neurite growth; however, a preliminary report (*41*) shows that a different organophosphate compound, chlorpyrifos (Dursban), decreases neurite outgrowth in cultured PC-12 cells. In human and animal studies, prenatal exposure to chlorpyrifos produces cellular and behavioral neurotoxicity (*42–44*); in the human study (*44*), CNS abnormalities were consistently noted, including structural defects in the ventricles and corpus callosum. Interestingly, young animals exhibit an increased susceptibility to organophosphorus insecticides (*45*), suggesting the necessity of further study to determine if these agents pose a teratogenic threat during critical periods of gestation. Because the developmental, morphogenic role for AChE in axonal growth is now well established, current research is directed toward understanding the mechanism of action and regulation of expression of this protein.

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